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Effect of insulin-like growth factor binding proteins on the response of proximal tubular cells to insulin-like growth factor-I

JASON YAP, TANNY TSAO, JANET FAWCETT, PAUL J. FIELDER, GILBERT A. KELLER, and RALPH RABKIN

Medicine Service Veterans Affairs Palo Alto Health Care System, Palo Alto; and Department of Stanford University School of Medicine, Stanford; and Genentech Inc., South San Francisco, California, USA

Effect of insulin-like growth factor binding proteins on the response of proximal tubular cells to insulin-like growth factor-I. The insulin-like growth factor binding proteins (IGFBP) are major modulators of insulin-like growth factor-I (IGF-I) action, but relatively little is known about their production by kidney tubular cells or about their modulating effects on the action of IGF-I on these cells. In this study we demonstrated that rabbit proximal tubular cells express the genes for IGFBP-2, -4 and -5 and secrete 24 and 32 kDa size binding proteins. The rate of IGFBP production by these cells was regulated by several growth factors including hydrocortisone, which was potently stimulatory, and EGF, which was inhibitory. The overall effect of these kidney cell-secreted IGFBPs was to inhibit the mitogenic activity of IGF-I. Similarly, recombinant IGFBP-3, the major circulating IGFBP that in kidney is produced close to the proximal tubules, also inhibited IGF-I stimulated DNA synthesis in cultured rabbit proximal tubular cells and in cultured opossum kidney (OK) cells. IGFBP-3 also inhibited basal DNA synthesis in OK cells in the absence of added IGF-I, suggesting that this IGFBP may have an IGF-I independent action. These findings highlight the important effect that IGFBPs have on the action of IGF-I on kidney cells and support the notion that the changes in IGFBPs observed in various renal diseases may contribute to the pathophysiology of these diseases.

Insulin-like growth factor-I (IGF-I) is a multifunctional growth factor produced in a variety of tissues including kidney [1, 2]. Its major renal actions include enhancement of tubular phosphate transport, renal blood flow, glomerular filtration rate and organ growth [2]. Indeed, IGF-I may participate in the induction of renal hypertrophy in several pathological conditions including diabetes mellitus, compensatory renal growth and hypokalemia [3]. The action of IGF-I is influenced by several IGF high-affinity binding proteins (IGFBPs) that are present in the circulation and are also produced locally throughout the body in a tissue specific pattern [1, 4]. Six IGFBPs have been identified [4]. In the circulation only 1 to 2% of the IGF-I is present in a freely bioavailable form [1, 5] and the balance is complexed to IGFBPs including IGFBPs-1 through -4. Of these IGFBP-3 is by far the most abundant and it accounts for > 90% of the complexed hormone in adult serum [4]. The overall effect of complex formation is a slowing of the passage of IGF-I into the tissues. In the tissues the IGFBPs originating locally or from the circulation are usually inhibitory [6], but there are reports that some IGFBPs may enhance IGF-I action [7–9]. This may arise through the trapping of IGF-I by cell associated IGFBPs with facilitation of the interaction between IGF-I and its receptor. Trapped IGF-I could also serve as a reservoir for the sustained release of the growth factor. Furthermore, IGFBPs may have cellular actions independent of IGF-I [1, 10, 11].

In kidney the genes for all six IGFBPs are detectable, though IGFBP-6 is present in low abundance [12, 13]. There is marked anatomical heterogeneity of expression of these genes in the kidney that suggests that the IGFBPs may have diverse functions. However, there is little information on the impact of tissue IGFBPs whether derived locally or from the circulation [14], on the renal actions of IGF-I [15, 16]. This may be of special importance to our understanding of disease states, for the changes in the IGFFBP profile that occur in a variety of kidney diseases may contribute to the pathophysiology of the disease by modifying the action of IGF-I [3, 17, 18].

In the present study we set out to characterize the IGFBPs produced by proximal tubular cells, determine the influence of growth factors on their production, and in turn discover the impact of the secreted IGFBPs on the mitogenic action of IGF-I. To this end rabbit proximal tubular cells were studied in primary culture. In addition, we examined the effect of recombinant IGFBP-3 on the action of IGF-I on these cells and on cultured opossum kidney (OK) cells, a cell line that has retained features of proximal tubular epithelium [19]. Insulin-like growth factor binding protein-3 was studied since *in vivo* the proximal tubules are likely exposed to tissue IGFBP-3 derived locally and from the circulation. This 43 to 53 kDa glycosylated protein is the most abundant circulating IGFBP and is also produced in close proximity to the proximal tubules in the peritublar compartment [12]

METHODS

Materials

Culture media was obtained from Gibco Laboratories (Grand Island, NY, USA); [^{3}H]-thymidine (0.2Ci/mmol), from Amersham Corp.; bovine serum albumin (BSA) from Armour Pharmaceutical Co. (Kankakee, IL, USA); and other chemicals, including bovine insulin and hydrocortisone from Sigma Chemical Co. (St. Louis, MO, USA). The following recombinant human proteins were provided by Genentech, Inc. (South San Francisco, CA,

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USA): IGF-I, des [1–3]IGF-I, glycosylated IGFBP-3, transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF) and growth hormone (GH). Epidermal growth factor (EGF) was purchased from Amgen Biologicals (Thousand Oaks, CA, USA).

Preparation of radiolabeled IGF-I

IGF-I was radiodinated by the chloramine T method as before and separated from free iodide by fractionation through a cellulose acetate column [15]. The specific activity of the ^{125}I-IGF-I was ~300 to 400 μCi/μg.

Cell cultures

Primary cultures of rabbit proximal tubular epithelial cells were isolated and cultured as previously described [19]. For these studies, monolayers were grown to confluency in one-half Dulbecco's modified Eagle's medium and one-half Ham's F-12 medium (DMEM/F12; 17.5 mM glucose) containing insulin (5 μg/ml), hydrocortisone (50 nM) and transferrin (5 μg/ml). Prior to study, the monolayers were deprived of insulin and hydrocortisone for 48 hours. At the time of the experiments this medium was refreshed with medium of the same composition containing 0.1% bovine serum albumin (BSA). In experiments examining the effects of growth factors or hydrocortisone, the monolayers were incubated for a further 24 hours with or without these additions present.

Opossum kidney (OK) cells from passages 40 to 50 were plated on 11.3 mm plastic tissue culture wells (~ 0.5×10^6 cells/well) in DMEM/F-12 containing 10% (vol/vol) newborn calf serum, penicillin (120 U/ml), and streptomycin (72 μg/ml) [15] and maintained at 37°C under an atmosphere of 5% CO_2-95% air. After reaching confluency, the medium was replaced with serum free medium of the same composition as before. Twenty-four hours later, at the time of the experiments, the serum free medium was refreshed with medium of the same composition containing 0.1% BSA and 10^{-9} M EGF. EGF was included since in preliminary experiments the presence of EGF enhanced IGF-I stimulated DNA synthesis.

Cell number

Cells were washed with calcium and magnesium free phosphate buffered saline, trypsinized, harvested and counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL, USA).

[^{3}H]-thymidine incorporation

Relative rates of DNA synthesis were determined from the incorporation of [^{3}H]-thymidine into tichloroacetic acid (TCA) insoluble material. Monolayers were incubated with 0.1 μCi [^{3}H]-thymidine per ml for four hours at 37°C and then washed with cold isotonic buffer and exposed to 10% TCA for 15 minutes. TCA insoluble material was solubilized in 0.1% SDS with 0.1 N NaOH and counted.

Charcoal adsorption assay

This was performed according to Concover et al [20]. In brief, conditioned medium was collected from confluent monolayers. Aliquots, 5 and 10 μl, were then incubated with ^{125}I-IGF (1×10^4 cpm) in 0.5 ml PBS with 0.5% BSA for 18 hours at 4°C. After incubation, 1 ml of 0.5% activated charcoal in PBS with 0.5% BSA and 0.2 mg/ml protamine sulfate was added and the mixture kept at 4°C for 45 minutes. Tubes were centrifuged at 3000 rpm for 30 minutes and the supernatant was separated from the charcoal precipitate. Radioactivity in the supernatant, which contained radioligand bound to binding proteins and the pellet, which contained free ^{125}I-IGF-I bound to charcoal, were counted in a gamma-counter. Unconditioned medium to which ^{125}I-IGF-I was added was assayed simultaneously to correct for the nonspecific appearance of free ^{125}I-IGF-I in the supernatant.

Western ligand blot

To measure secreted IGFBPs, 48-hour conditioned media was concentrated tenfold by centrifugation in Centricon-10 concentrators (Amicon Inc., Beverly, MA, USA) and then analyzed according to Hossensloop et al [21]. In brief an aliquot of the concentrate was dissolved in Laemmli's sample buffer, heated to 95°C for five minutes and electrophoresed in a 12% SDS-polyacrylamide gel. The separated proteins were electroblotted onto nitrocellulose filters. These were blocked with BSA and Tween-20 and probed with ^{125}I-IGF-I (2×10^6 cpm) overnight at 4°C. The filter was washed with Tris-buffered saline, air-dried and exposed to Kodak XAR X-ray film for two to four days. The signal was measured by densitometry (Gel Scan XL; Pharmacia LKB, Alameda, CA, USA). It should be recognized that this is not a truly quantitative assay because of variations in the transfer of the IGFBPs to the nitrocellulose.

Northern blot analysis

For each analysis total mRNA was extracted from three or more confluent monolayers of rabbit proximal tubular cells grown in 10 cm diameter culture wells. This was achieved by the single step method of Chomcznyski and Sacchi [22], using acid guanidinium thiocyanate-phenol chloroform extraction and the RNA was quantitated by absorbance at 260 nm. For Northern analysis of IGFBP mRNA levels, 12 μg of total RNA was fractionated on a 0.8% denatured agarose gel and the ethidium bromide stained-RNA photographed under UV light. RNA was transferred to a nitrocellulose membrane overnight and hybridized with [^{32}P]d-CTP labeled rat IGFBP-1, -2, -3, -4, and -5 cDNA probes as previously described. To control for loading the filter was also probed with radiolabeled human GAPDH cDNA [18].

Densitometric analysis

Radioactive and chemiluminescent signals captured on X-ray films were quantitated by laser densitometry (GelscanXL; Pharmacia LKB, Alameda, CA, USA).

Statistics

The results are expressed as the mean ± SEM. For comparisons between two groups the data were analyzed with Student's t-test, and a P value of < 0.05 was taken as significant. When three or more (up to 5) groups were compared an analysis of variance was used. If the F ratio was significant this was followed by Student's t-test with Bonferronis adjustment for multiple comparison, and a P value of < 0.01 was taken as significant [23].

RESULTS

Cultured primary rabbit proximal tubular cells

IGFBP genes are expressed by rabbit proximal tubular cells. Messenger RNA extracted from cultured rabbit proximal tubular cells and subjected to Northern analysis with rat IGFBP-1 to -5

Table 1. Effect of growth factors and hydrocortisone on IGFBP mRNA levels in cultured rabbit proximal tubular cells

	Treatment				
	GH (50 nm)	TGF-β (4 nM)	EGF (10 nM)	HGF (10 nM)	Hydrocortisone (1.4 μM)
IGFBP-2	193 ± 42.7	64 ± 12.5[b]	19 ± 1.2[b]	35 ± 4.0[b]	110 ± 12.0
IGFBP-4	134 ± 15.2	73 ± 4.5[b]	23 ± 5.3[b]	48 ± 10.7[b]	394 ± 124.8
IGFBP-5	144 ± 29.5	71 ± 11.5	117 ± 14.5	65 ± 11.0[a]	25 ± 16.0[b]

IGFBP mRNA levels were detected by Northern blot analysis and corrected for the corresponding GAPDH mRNA level. Results, mean ± SEM, are from 3 to 6 separate experiments and are expressed as a percentage of the corresponding controls. Exposure to treatment was for 24 hours.
[a] $P < 0.05$, [b] $P < 0.01$ versus control values

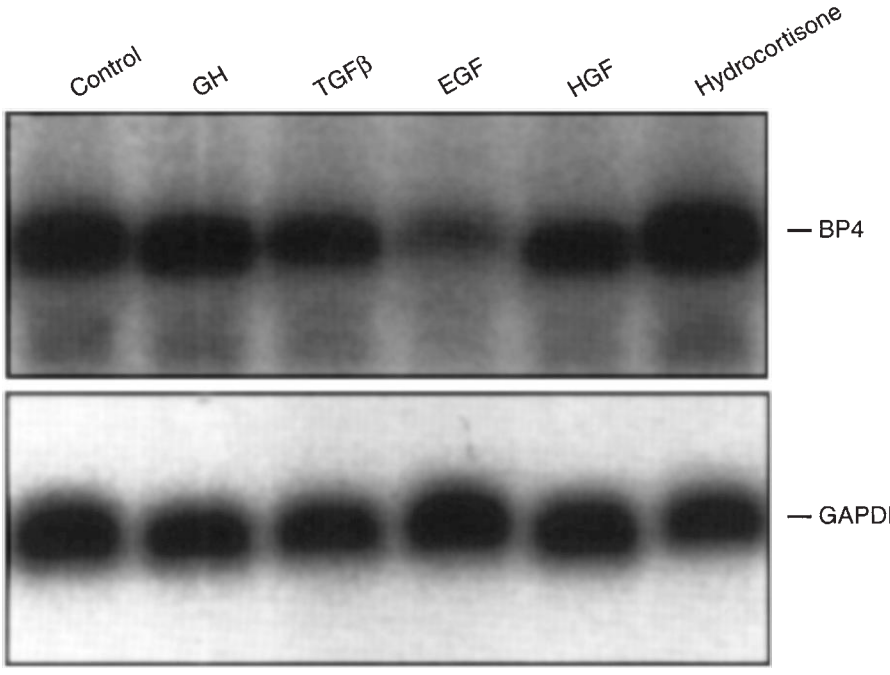

Fig. 1. Northern blot analysis of IGFBP-4 and GAPDH mRNA levels in cultured proximal tubular cells. Cells were incubated in the presence or abscence of the indicated growth factors for 24 hours at the concentrations listed in Table 1. The autoradiographs are from a single gel.

cDNA probes gave positive signals for IGFBP-2, -4 and -5 only. The effect of a variety of growth factors and hydrocortisone on the expression of these mRNAs are summarized in Table 1 and an example of a Northern blot analysis is shown in Figure 1. Incubation with TGF-β, EGF and HGF, growth factors expressed in kidney, decreased the IGFBP-2 and -4 mRNA levels significantly while hydrocortisone increased the IGFBP-4 mRNA level fourfold. IGFBP-5 mRNA levels were depressed significantly by HGF and hydrocortisone. GAPDH mRNA levels were not affected by any of the treatments.

Western ligand blot of IGFBPs secreted into the culture medium. When culture medium conditioned for 48 hours was analyzed by Western ligand blotting with ^{125}I-IGF-I, two distinct bands of IGF-I binding activity were detected (Fig. 2). These were ~24 and 32 kDa in size and are most consistent in size with IGFBP-4 (24 kDa) and IGFBP-2 or -5 (~32 kDa). Similar size proteins are present in rabbit serum [24].

Regulation of IGFBP secretion by growth factors. Net IGFBP secretion into the culture medium over 24 hours was quantitated by a charcoal adsorption assay with ^{125}I-IGF-I as the ligand. The effects of GH, TGFβ, EGF, HGF, hydrocortisone and insulin are shown in Figure 3. TGFβ and EGF each produced a modest, but significant depression of IGFBP secretion (13 and 34%, respectively). In contrast, GH stimulated a small (15%), but significant increase in IGFBP secretion while hydrocortisone increased secretion by 125% ($P < 0.01$); insulin had no effect. IGF-I was not examined as it interferes with the charcoal assay.

IGF-I stimulates [^{3}H]-thymidine incorporation and cell proliferation. In these experiments confluent cultured proximal tubular cells were exposed to IGF-I or insulin, 10^{-9} to 10^{-6} M, in serum free medium for 24 hours to study DNA synthesis and 48 hours to study cell proliferation. As depicted in Figure 4, IGF-I stimulated DNA synthesis and cell proliferation more effectively than insulin. At IGF-I concentrations of 10^{-8} M or above, [^{3}H]thymidine incorporation increased significantly to values two- to threefold greater than that seen in untreated control cells. A similar response was seen with insulin at concentrations an order of magnitude higher, 10^{-7} to 10^{-6} M. The IGF-I induced proliferative response mirrored DNA synthesis. IGF-I at concentrations of 10^{-8} M or above stimulated a significant increase in cell number

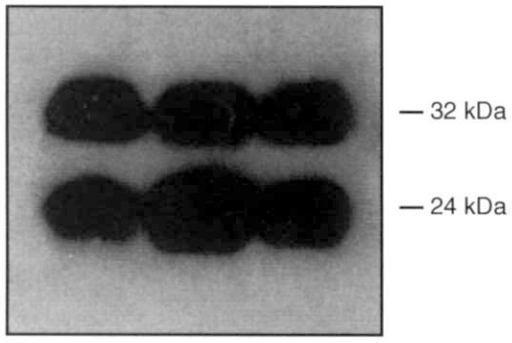

Fig. 2. Western ligand blot of IGFBPs secreted by cultured rabbit proximal tubular cells. The autoradiogram was obtained with 48-hour conditioned culture medium; ^{125}I-IGF-I was used as the ligand.

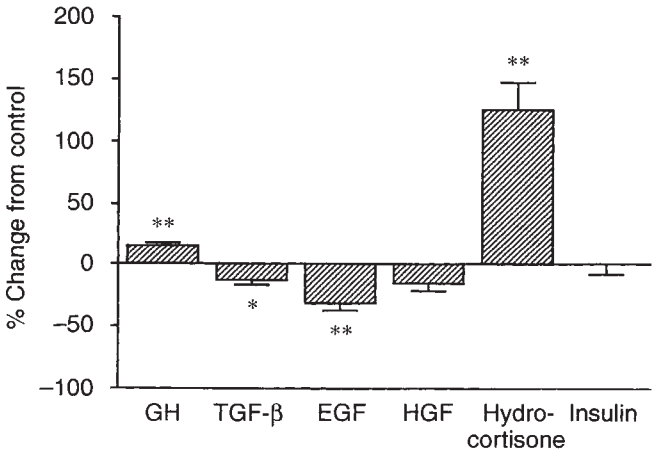

Fig. 3. Effect of growth factors on net IGFBP production by rabbit proximal tubular cells. Cells were incubated in the presence or absence (controls) of growth factors for 24 hours. The following concentrations of growth factors were added: GH 50 nM, TGFβ 4 nm, EGF 10 nm, HGF 10 nm and hydrocortisone 1.4 μM. Conditioned medium was assayed by means of the charcoal binding assay with ^{125}I-IGF-I as the ligand. The results are the mean ± SEM of 5 to 11 seperate experiments and are expressed as the net percentage change in the treatment groups compared to untreated controls. $^{*}P < 0.05$, $^{**}P < 0.01$

while insulin was only effective at 10^{-6} M. This proliferative response to insulin is consistent with insulin cross-reacting with and stimulating the IGF-I receptor.

Secreted IGFBPs inhibit IGF-I stimulated DNA synthesis. To evaluate the effect of IGFBPs secreted by the rabbit kidney cells on the action of IGF-I, we compared the dose response to IGF-I in conditioned medium with that of des(1-3)IGF-I, a truncated analog that binds to the IGF-I receptor with the same affinity as the full length molecule [18], but binds poorly to IGFBPs [25]. In competitive binding experiments, we confirmed that this later property also holds true for rabbit kidney cell IGFBPs. These experiments (data not shown) revealed that the affinity of rabbit kidney cell IGFBPs for des(1-3)IGF-I was reduced by more than an order of magnitude compared to IGF-1. The half maximal concentration of cold IGF-I or des(1-3)IGF-I that displaced 50% of the tracer ^{125}I-IGF-I bound to the kidney cell IGFBPs, a measure of relative affinity, averaged 1.6×10^{-10} M for IGF-I and 6×10^{-9} M for des(1-3)IGF-I, respectively. Next we incubated proximal tubular cell monolayers with 48-hour conditioned serum-free medium containing increasing concentrations of IGF-I or des(1-3)IGF-I and measured [^{3}H]-thymidine incorporation. As shown in Figure 5 at concentrations below 10^{-8} M, des(1-3)IGF-I stimulated [^{3}H]-thymidine incorporation to a significantly greater extent than equimolar concentrations of IGF-I. Indeed, at 10^{-10} M IGF-I had no effect on ^{3}H]-thymidine incorporation, while des(1-3)IGF-I stimulated incorporation by 80% above the contol value. This indicates that IGFBPs secreted by cultured rabbit proximal tubular cells have a net inhibitory effect on IGF-I stimulated DNA synthesis.

Recombinant IGFBP-3 inhibits IGF-I stimulated DNA synthesis and proliferation. In these experiments [^{3}H]-thymidine incorporation was measured after exposing rabbit proximal tubular cells to 10^{-8} M IGF-I for 24 hours in the presence of an equimolar concentration of recombinant glycosylated human IGFBP-3 [26–28] or vehicle (control). Monolayers were placed in fresh medium containing IGFBP-3 and two hours later IGF-I was added; IGFBP-3 was present throughout the subsequent incubation. As shown in Table 2, IGFBP-3 inhibited IGF-I stimulated [^{3}H]-thymidine incorporation significntly ($P < 0.01$). In other monolayers the cell number was measured after 48 hours of exposure to IGF-I. As occurred with DNA synthesis, IGF-I stimulated cell proliferation was inhibited when IGFBP-3 was present ($P < 0.05$). Recombinant IGFBP-3 added to monolayers in the absence of IGF-I had no effect on DNA synthesis or cell proliferation.

Cultured opossum kidney cells

IGF-I stimulated DNA synthesis and this was inhibited by free but not cell associated IGFBP-3. Addition of 10^{-8} M IGF-I to confluent OK cell monolayers incubated in serum free culture medium stimulated [^{3}H]-thymidine by 26% ($P < 0.01$) compared to vehicle treated controls. This is shown in Figure 6, which also shows the effect of recombinant IGFBP-3 on basal and IGF-I stimulated DNA synthesis. When OK cells were incubated with IGFBP-3 alone basal [^{3}H]thymidine incorporation decreased significantly by 29% (Fig. 6A). When cells were coincubated with IGF-I (10 nM) and increasing concentrations of IGFBP-3, growth factor stimulated ^{3}H]-thymidine incorporation was completely inhibited at an IGFBP-3 concentration of 50 nM or above (Fig. 6B).

Since it is conceivable that OK cells may produce IGF-I, we considered the possibility that the IGFBP-3 induced attenuation of basal DNA synthesis might reflect the inhibition of endogenous IGF-I. To test this concept, control monolayers incubated in serum free medium were exposed to a neutralizing IGF-I antibody (Upstate Biotechnology Inc., Lake Placid, NY, USA). At an antibody concentration that completely blocks the stimulation of DNA synthesis by 10^{-8} M IGF-I in OK cells there was a statistically insignificant decrease in DNA synthesis averaging 10%. This argues against significant OK cell IGF-I production and suggests that IGFBP-3 inhibits DNA synthesis through an IGF-I independent action.

It has been shown that prolonged incubation of cultured fibroblasts with IGFBP-3 can enhance their responsiveness to IGF-I [7, 8]. This has been attributed to an increase in cell surface

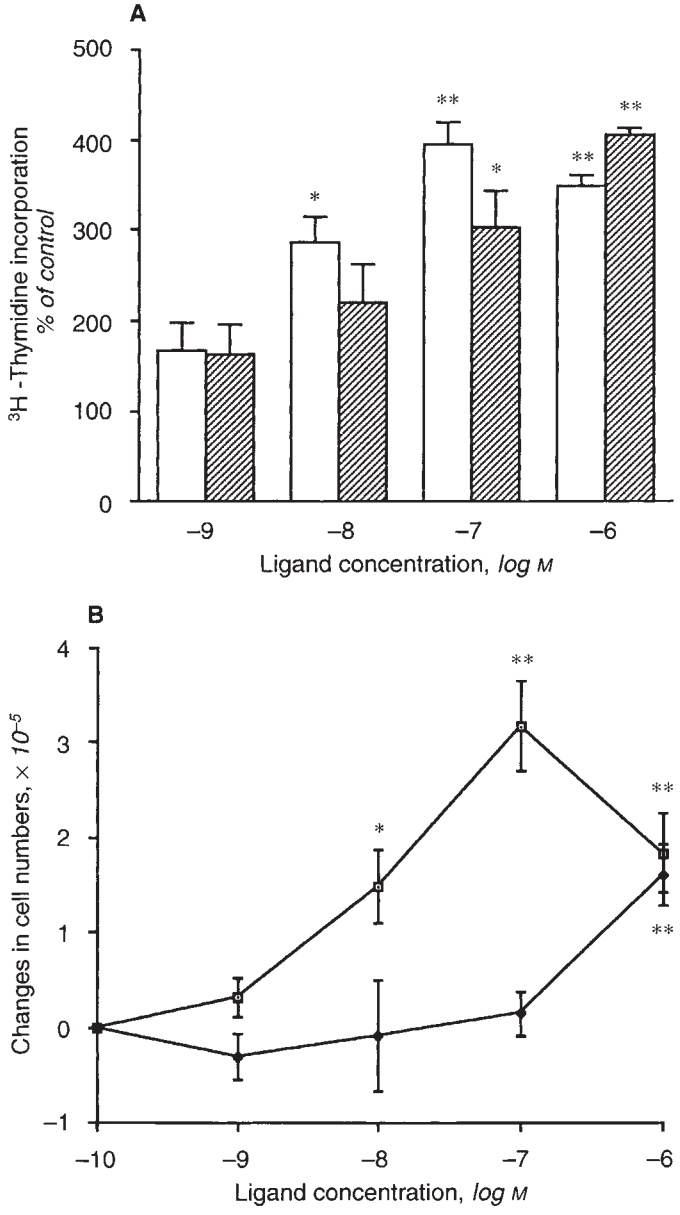

Fig. 4. Effect of IGF-I and insulin on [^{3}H]thymidine incorporation (*A*) and cell proliferation (*B*). Cultured rabbit proximal tubule cells were incubated in serum free medium containing 10^{-8} M IGF-I or insulin for 24 hours for measurement of [^{3}H]-thymidine incorporation ($N = 3$) and 48 hours for measurement of cell number ($N = 8$ separate experiments). Symbols are (□, ⊡) IGF-I; (▨, ◆) insulin. $^*P < 0.05$, $^{**}P < 0.01$ compared to untreated controls.

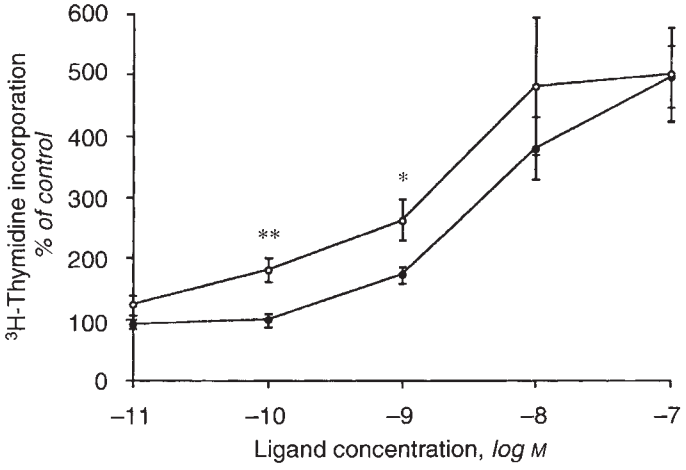

Fig. 5. Effect of IGF-I (●) and des(1-3)IGF-I (○) on [^{3}H]-thymidine incorporation in cultured rabbit proximal tubular cells in the presence of endogenous IGFBPs. Confluent rabbit proximal tubular cell monolayers were incubated for 24 hours in 48-hour conditioned medium containing increasing concentrations of IGF-I or des(1-3)IGF-I. $^*P < 0.05$, $^{**}P < 0.01$ compared to controls ($N = 4$ separate experiments).

associated IGFBP-3 with increased trapping of IGF-I and exposure to the IGF-I receptor. The following experiment tested whether cell associated IGFBP-3, as opposed to free IGFBP-3 in the experiments above, increases the association of IGF-I with kidney cells and thereby enhances its action. OK cells were preincubated with 10^{-8} M IGFBP-3 for 24 or 72 hours, the medium was replaced with fresh IGFBP-3 free medium containing ^{125}I-IGF-I (2×10^{-10} M). As shown in Figure 7, preincubation with IGFBP-3 for 72 hours produced a small but significant increase in the total amount of cell associated [^{125}I]IGF-I compared to vehicle incubated controls (10.8 ± 0.3 vs. 9.5 ± 0.4%/monolayer, respectively, $P < 0.01$). To differentiate between increased binding to IGF-I receptors or cell associated IGFBP-3, displacement by 10^{-4} M cold insulin was examined. Insulin at high concentrates competes with IGF-I for the IGF-I receptor but not for any binding proteins. In the presence of 10^{-4} M cold insulin, the residual radioactivity associated with IGFBP-3 pretreated cells was significantly greater than the radioactivity associated with the contol cells (1.88 ± 0.13 vs. 0.66 ± 0.03%, respectively, $P < 0.001$). Note that the difference in residual cell associated radioactivity (1.22%) between the two groups was comparable to the difference in total cell associated ^{125}I-IGF-I (1.3%). This indicates that the increase in ^{125}I-IGF-I binding to IGFBP-3 pretreated cells reflects sequestration of ^{125}I-IGF-I by cell associated IGFBP-3. However, unlike fibroblasts [7], the IGFBP-3 mediated increase in OK cell associated IGF-I did not enhance IGF-I stimulated DNA synthesis. In cells preincubated with IGFBP-3 or vehicle for 72 hours [^{3}H]thymidine incorporation averaged 2739 ± 811 versus 2532 ± 847 dpm/well, respectively. In cells preincubated with IGFBP-3 or vehicle for 24 hours [^{3}H]-thymidine incorporation averaged 2190 ± 298 versus 2024 ± 196 dpm/well, respectively.

DISCUSSION

This study demonstrates that rabbit proximal tubular cells grown in primary culture secrete IGFBPs that inhibit the mitogenic action of IGF-I. Northern blot analysis showed that these cells express IGFBP-2, -4 and -5 mRNAs and Western ligand blot analysis of culture medium showed that they secrete IGFBPs of molecular weights ~28 and 32 kDa. Similarly sized IGFBPs are present in rabbit serum [24]. The regulation of IGFBP secretion was quantified with a charcoal adsorption assay and we found that TGFβ and EGF, growth factors produced within the kidney, reduced secretion modestly; growth hormone produced a minor increase, while hydrocortisone stimulated secretion twofold. As reported with IGFBPs from other animal species [25], the affinity

Table 2. Effect of IGFBP-3 on IGF-1 stimulated DNA synthesis and cell proliferation in rabbit proximal tubular cells

	Control	IGFBP-3	IGF-1	IGF-1 + IGFBP-3
[^{3}H]-thymidine incorporation *dpm*, $\times 10^{-3}$	7.96 ± 1.10	7.18 ± 0.59	25.01 ± 3.28[b]	17.92 ± 31.0[b]
Cell number $\times 10^{-5}$	11.52 ± 1.18	11.72 ± 1.13	12.76 ± 1.41[a]	11.95 ± 1.51

DNA synthesis measured from incorporation of [^{3}H]-thymidine after 24 hours exposure to test substance. Cell proliferation was measured after 48 hours. IGF-1 and IGFBP-3 were present at 10^{-8} M.
[a] $P < 0.05$, [b] $P < 0.01$ vs. all other groups

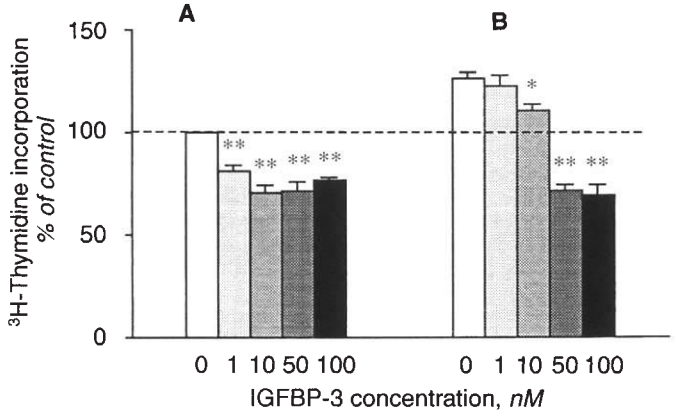

Fig. 6. Effect of the presence of IGFBP-3 in the culture medium on (*A*) basal and (*B*) IGF-I stimulated [^{3}H]-thymidine incorporation in OK cells. Increasing concentrations of IGFBP-3 were added to confluent OK cells incubated in serum free medium two hours before the addition of vehicle (A) or 10 nM IGF-I (B). [^{3}H]-thymidine incorporation was measured 24 hours later. $*P < 0.05$, $**P < 0.01$ compared to respective IGFBP-3 free controls ($N = 4$ separate experiments).

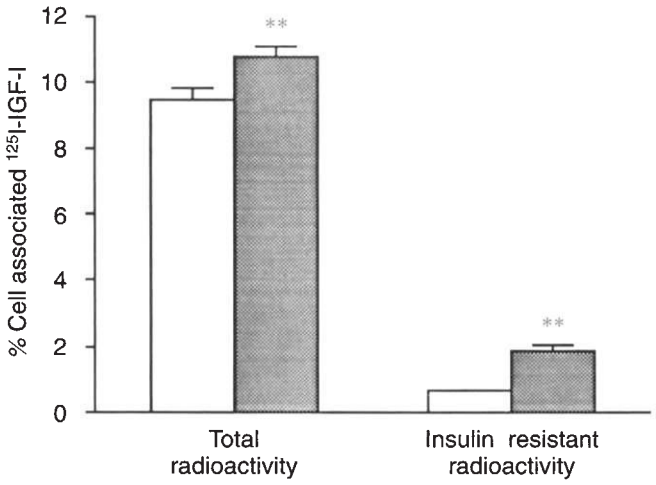

Fig. 7. Effect of preincubating cultured OK cells with IGFBP-3 for 72 hours for the cellular association of ^{125}I–IGF-I. Monolayers were incubated with IGFBP-3 (□) or vehicle (▩) for 72 hours, washed twice and then incubated in fresh IGFBP-3 free medium containing ^{125}I-IGF-I (2×10^{-10} M) with or without 10^{-4} M insulin for 60 minutes at 37°C. Radioactivity displaced by insulin is taken to reflect binding to receptors. The difference in cell associated radioactivity between the two groups is taken to reflect ^{125}IGF-I bound to cell associated IGFBP-3 ($N = 5$ experiments/group). $**P < 0.01$.

of these secreted IGFBPs for IGF-I was considerably greater than for the truncated analog, des(1-3)IGF-I. In the presence of rabbit cell IGFBPs, IGF-I stimulated the synthesis of DNA less effectively than des(1-3)IGF-I. This is consistent with a reduction in IGF-I availability due to sequestration by the secreted IGFBPs.

Next we examined the effect of IGFBP-3 on the action of IGF-I since *in vivo* it is likely that the proximal tubules are exposed to IGFBP-3 derived from the circulation and also to IGFBP-3 produced locally in the cortical peritubular compartment [12, 13]. We found that when rabbit proximal tubular cells were coincubated with IGF-I and recombinant IGFBP-3, IGF-I-stimulated DNA synthesis and cell proliferation were inhibited. Similarly, IGFBP-3 inhibited DNA synthesis in cultured OK cells. These findings are consistent with studies conducted with cultured human mesangial cells [16] and cells derived from other tissues [9, 10, 26]. It is likely that inhibition is a consequence of IGF-I-IGFBP-3 complex formation that limits the bioavailability of IGF-I. Indeed, IGFBP-3 in solution exhibits a significantly greater affinity for IGF-I than the IGF-I receptor [1]. Furthermore, in an earlier study with OK cells we showed directly that IGFBP-3 inhibits IGF-I receptor binding and receptor mediated internalization significantly [15].

In addition to inhibiting IGF-I action by reducing its bioavailability, IGFBP-3 may have a direct IGF-I independent inhibitory action. This has been described with IGFBP-3 transfected fibroblasts [11] and with a cultured breast cancer cell line [10], and may possibly be mediated through IGFBP-3 specific receptors [27]. In the present study we did find evidence that indicates that IGFBP-3 may have an IGF-I independent action on cultured OK cells, but not rabbit tubular cells. Exposure of OK cells to IGFBP-3 in the absence of exogenous IGF-I inhibited DNA synthesis significantly by 30%. This was not mediated through the inhibition of any IGF-I that might be produced by OK cells, for the inhibitory effect of IGFBP-3 could not be reproduced with an excess of IGF-I specific blocking antibody. A potential mechanism for the IGF-I independent action is suggested from a recent study from this laboratory, which demonstrated that IGFBP-3 targets to the nucleus of OK cells [28]. It is conceivable that the IGFBP-3 arriving in the nucleus directly inhibited DNA synthesis at this site.

Adding to the complexity of the action of IGFBP-3 are reports that IGFBP-3 may actually enhance the action of IGF-I in cultured cells such as fibroblasts and osteoblasts [7–9, 30]. In some studies the enhancing effect was only observed when cells were preincubated for 24 to 48 hours with IGFBP-3 and then, after removing the protein from the incubation medium, the cells were exposed to IGF-I [8, 9, 29]. This enhancing effect was thought to be mediated through an increase in cell associated IGFBP-3 that trapped and presented IGF-I to its receptors. In the present study when OK cells were preincubated with IGFBP-3 for periods up to

72 hours, cell associated IGFBP-3 increased significantly. However, unlike the studies described above, when the cells were subsequently treated with IGF-I there was no enhancement or inhibition of IGF-I action.

The situation *in vivo* is of course considerably more complicated than in cell culture. Also, responses observed *in vitro*, especially in cell lines, may be acquired under cell culture conditions. Accordingly, despite the novel information derived from cells in culture, caution must be exercised when extrapolating these data to the situation *in vivo*. For example, while the immediate effect of IGFBPs secreted by cultured proximal tubular cells are inhibitory, it is conceivable that *in vivo* sequestration of IGF-I by IGFBPs within the kidney could result in an elevation of local IGF-I levels [3]. In turn, the slow and sustained release of free IGF-I from this pool, especially when IGF-I production is reduced, could increase the interaction of IGF-I with the IGF-I receptor and thus facilitate IGF-I activity [1, 31]. Such a process has been postulated to occur in diabetes and in K depletion [3, 32]. Early in both these conditions, kidney IGF-I levels increase even though in diabetes the mRNA levels are unchanged, while in K depletion the levels are actually reduced. The increase in IGF-I has been attributed in part to elevated local IGFBP levels and may contribute to the renal hypertrophy that occurs in diabetes and in K depletion [3, 32]. From all this, it is apparent that our understanding of the action of the IGFBPs in the kidney is very inadquate. Nevertheless it seems reasonable to propose that the IGFBPs play a role in the pathophysiology of certain renal diseases by altering the local IGF-I levels and the interaction of IGF-I with its receptor.

In summary, we have shown that cultured rabbit proximal tubular cells produce IGFBPs and that their rate of production is inhibited by several growth factors, especially EGF, and stimulated by others, especially hydrocortisone. These endogenous IGFBPs have a net inhibitory effect on IGF-I stimulated DNA synthesis. Similarly, the recombinant form of glycosylated IGFBP-3, the major circulating IGF binding protein, which in kidney is produced in cells close to the proximal tubule, also inhibits IGF-I stimulated DNA synthesis in cultured rabbit and OK cells. Furthermore, it appears that in OK cells IGFBP-3 may have an IGF-I-independent inhibitory action. Since in a variety of kidney disease states there are marked changes in the circulating and local kidney IGFBP profile, it may well be that these changes contribute to the pathophysiologic process by influencing the action of IGF-I and perhaps also through an IGF-I independent action. Unravelling the contribution of the various components of the IGF-I–IGFBP system remains an important challenge.

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Reprint requests to Ralph Rabkin, M.D., VAMC Palo Alto (154L), 3801 Miranda Avenue, Palo Alto, California 94304, USA.
E-mail: rabkin@leland.stanford.edu

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